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14. ABSTRACT

Fragile X Syndrome (FXS) is a single gene disorder caused by loss of *FMR1* gene function. This disease leads to cognitive impairment and is the most common genetic cause of autism, accounting for 2-6% of all diagnosed cases (Hagerman et al 2008). In previous studies of a *Drosophila* model for FXS, we identified pharmacological treatments that rescued phenotypes relevant to this syndrome such as social, neuroanatomical and cognitive deficits (McBride et al., 2005; Choi et al., 2010). These results have been translated to the mouse model of FXS leading to the impetus to initiate clinical trials with Fragile X patients (Yan et al., 2005; Dolen et al., 2007; de Vrij et al., 2008; Choi et al., 2011). The fact that clinical trials of two distinct compounds identified in flies and tested in mice have reported some level of efficacy highlights the relevance of *Drosophila* and mouse-based disease modeling to identify potential treatments for developmental brain disorders and other diseases (Berry-Kravis et al., 2008; Berry-Kravis et al., 2009; Jacquemont et al., 2011). Our objective is to fully explore a recently defined link between metabotropic glutamate receptor (mGluR) signaling and the mTOR pathway, two pathways that have been previously examined in Fragile X without having the pathways involving these two proteins dissected in depth (Banko et al., 2006; Ronesi and Huber, 2008; Sharma et al., 2010). In preliminary testing of this link we have identified additional pharmacologic treatments that rescue either the cognitive and/or social interaction deficits displayed by the *Drosophila* model (TAJ and SmcB, unpub.). Our objective is to fully explore the link between these two pathways to identify as many potential targets for pharmacological intervention of FXS. Since several of the genes that link these

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Introduction

Fragile X syndrome is the leading cause of intellectual disability resulting from a single gene mutation. Previously, we characterized social and cognitive impairments in a *Drosophila* model of Fragile X syndrome and demonstrated that these impairments were rescued by treatment with metabotropic glutamate receptor (mGluR) antagonists or lithium. In the mouse model of Fragile X a well-characterized phenotype is enhanced mGluR-dependent long-term depression (LTD) at Schaffer collateral to CA1 pyramidal synapses of the hippocampus. Herein, we have now identified a novel drug target in the mGluR signaling pathway, phosphodiesterase-4 (PDE-4), and demonstrate PDE-4 inhibition as a therapeutic strategy to ameliorate memory impairments in the *Drosophila* model of Fragile X. Furthermore, we examine the effects of PDE-4 inhibition by pharmacologic treatment in the Fragile X mouse model. Acute inhibition of PDE-4 by pharmacologic treatment in hippocampal slices rescues the enhanced mGluR-dependent LTD phenotype. Additionally, chronic treatment of Fragile X mice in adulthood with a PDE-4 inhibitor for eight weeks also restores the level of mGluR-dependent LTD to those observed in wild type (WT) animals. Translating the findings of successful pharmacologic intervention from the *Drosophila* model into the mouse model of Fragile X syndrome is an important advance, in that this identifies and validates PDE-4 inhibition as potential therapeutic intervention for the treatment of individuals afflicted with Fragile X syndrome.

A) Completion of PDE4 studies in the *Drosophila* fragile X model. (items 1a, 1b,part of 13a and 13d on Statement of Work.

The *Drosophila* model of Fragile X is based on loss of function of *dfmr1*, the *Drosophila* orthologue of *FMR1*. For our studies, we use a *dfmr1* deletion line carrying a genomic transgene with a frame shift mutation engineered in the *dfmr1* coding region that is driven by the endogenous promoter, referred to as the FS line. The control line for these studies contains the same deletion of the *dfmr1*gene, but also carries a wild type transgene for *dfmr1* that is driven by the endogenous promoter and is referred to as the WT line (Dockendorff et al., 2002; McBride et al., 2005).

In *Drosophila*, cognitive ability can be assessed utilizing the conditioned courtship associative memory paradigm. A male fly will display a semi-stereotyped set of courtship behaviors when paired with a female. These behaviors can be scored and the percentage of time spent engaged in these courtship behaviors during a testing period is referred to as a courtship index (CI) (Siegel and Hall, 1979). If a male is paired with a previously mated female over the course of one hour, his courtship will decrease during the training period due to the female's aversive cues and rejection of his advances. This decrease in courtship during the training period is referred to as learning during training (LDT) (Joiner Ml and Griffith, 1997; Kane et al., 1997). Additionally, the male will continue to have lower courtship activity when subsequently paired with a virgin female, compared to males that are not paired with a previously mated female. This lower courtship activity is indicative of a memory of the training. An alternative version of this paradigm pairs the trained male with a novel previously mated female target after training (Siegel and Hall, 1979; Kamyshev et al., 1999; McBride et al., 2005). The comparison is then between the courtship index (CI) during the initial 10 minute period of training and the CI during the testing period (Kamyshev et al., 1999; McBride et al., 2005). Again a reduction in CI during the testing period is indicative of memory. Males can be tested immediately after training to assess immediate recall memory or 60 minutes after training to assess short-term memory.

FS flies have been demonstrated to have impairments in immediate recall, short-term memory and long-term memory in the conditioned courtship paradigm (McBride et al., 2005; Banerjee et al., 2010; Choi et al., 2010). We chose to inhibit the *Drosophila* PDE-4, which hydrolyzes cAMP, with the pharmacologic inhibitors rolipram and Ro-20-1724. We hypothesized that PDE-4 inhibition would rescue memory by correcting the over-active mGluR signaling in the Fragile X fly model resulting in decreased cAMP levels after stimulation due to inhibition of PDE-4 by rescuing cAMP levels (Figure 1A) (McBride et al., 2005). PDE-4/*dunce* was first identified as a memory mutant in *Drosophila* and later the orthologue was subsequently cloned in mammals (Dudai et al., 1976; Byers et al., 1981; Davis et al., 1989). Previous studies have demonstrated decreased cAMP levels in cells taken from Fragile X patients and Fragile X animal models, as well as a positive correlation between FMRP levels and cAMP levels in cell lines (Berry-Kravis and Huttenlocher, 1992; Berry-Kravis et al., 1995; Berry-Kravis and Ciurlionis, 1998; Kelley et al., 2007). Rolipram has been demonstrated to have efficacy in *Drosophila* at doses higher than those used in earlier studies (Henkel-Tigges and Davis, 1990;

Hou et al., 2004). Rolipram has also been demonstrated to increase CREB mediated gene transcription in *Drosophila* (Hou et al., 2004). Thus it may be able to partially circumvent the mGluR mediated inhibition of cAMP signaling incurred in Fragile X cells.

In order to test the hypothesis that PDE-4 inhibition may rescue cognitive impairments in Fragile X flies, FS and WT flies were treated with rolipram, Ro-20-1724 or the appropriate vehicle for 9 days (starting on the first day of eclosion) and then tested for immediate recall (0-minute memory) and short-term memory (60-minute memory) as well as LDT and short-term memory in an alternative memory paradigm that utilizes a previously mated target female. Immediate recall memory and short-term memory impairments have been previously demonstrated in the FS flies (McBride et al., 2005; Bolduc et al., 2008; Banerjee et al., 2010; Choi et al., 2010). FS flies demonstrated rescued immediate recall memory and short-term memory after treatment with both PDE-4 inhibitors. In contrast FS flies continued to have impaired immediate recall memory and short-term memory when treated with the vehicle controls for rolipram and Ro-20-1724 (Fig.1B and C). WT flies displayed intact immediate recall memory or short-term memory when treated with PDE-4 inhibitors or vehicle (Fig. 1B and C). FS and WT flies displayed intact LDT regardless of treatment (Fig. 1 D). LDT in young adult FS flies has been previously demonstrated to be intact, and our results demonstrate that PDE-4 inhibition does not impair LDT in FS flies (Fig. 1D) (McBride et al., 2005; Choi et al., 2010). Treatment with either the PDE-4 inhibitor rolipram or Ro-20-1724 rescued short-term memory in the alternative memory paradigm in FS flies, whereas vehicle treatment did not (Fig. 1D). WT flies displayed intact short-term memory on vehicle or PDE-4 inhibitor treatments (Fig. 1D). We next wanted to genetically validate the specificity of the PDE-4 inhibitor treatments by crossing in the *dunce* mutation into the Fragile X background. The dunce mutation is a loss of function mutation of the PDE-4 gene, resulting in abnormally high cAMP levels and memory impairment (Byers et al., 1981; Davis and Kiger, 1981). We found that Fragile X flies carrying the dunce mutation demonstrated rescued short-term memory in the standard and alternative memory paradigms (Fig. 1E and F), thereby confirming PDE-4 as a potential therapeutic drug target for the amelioration of cognitive impairment displayed in Fragile X.

The mushroom bodies are a structure in the insect brain that was first speculated to be involved in memory by having an analogous structure to the human hippocampus and is currently often regarded as the analogous structure in the fly (Dujardin, 1850; O'Kane, 2011). The mushroom bodies were demonstrated to be required for short-term and long-term memory in the conditioned courtship paradigm (McBride et al., 1999) and the olfactory-based paradigm (Zars et al., 2000; Pascual and Preat, 2001). Fragile X model flies exhibit a phenotype of aberrant midline crossing of the beta lobes of the mushroom bodies, which is corrected by treatment with mGluR antagonists or lithium (McBride et al., 2005). The PDE-4 inhibitor, rolipram, at the treatment dose that rescued memory did not rescue the phenotype of aberrant midline crossing by the beta lobes of the mushroom bodies in the brains of FS flies (not shown). However a higher dose of rolipram at 500mM did rescue the phenotype of aberrant midline crossing by the beta lobes of the mushroom bodies in the brains of FS flies, whereas vehicle treatment had no effect (Fig. 1G). This result left us with two possible explanations of how the higher dose that rescues the midline crossing defect would affect memory, it could make it worse or it could continue to rescue memory. We then re-examined the memory of FS flies with this higher dose of rolipram. We found that the higher dose of rolipram continued to demonstrate efficacy in the rescue of short-term memory in both the standard and alternative short-term memory paradigms, whereas vehicle treatment did not (Fig. 1H and I).

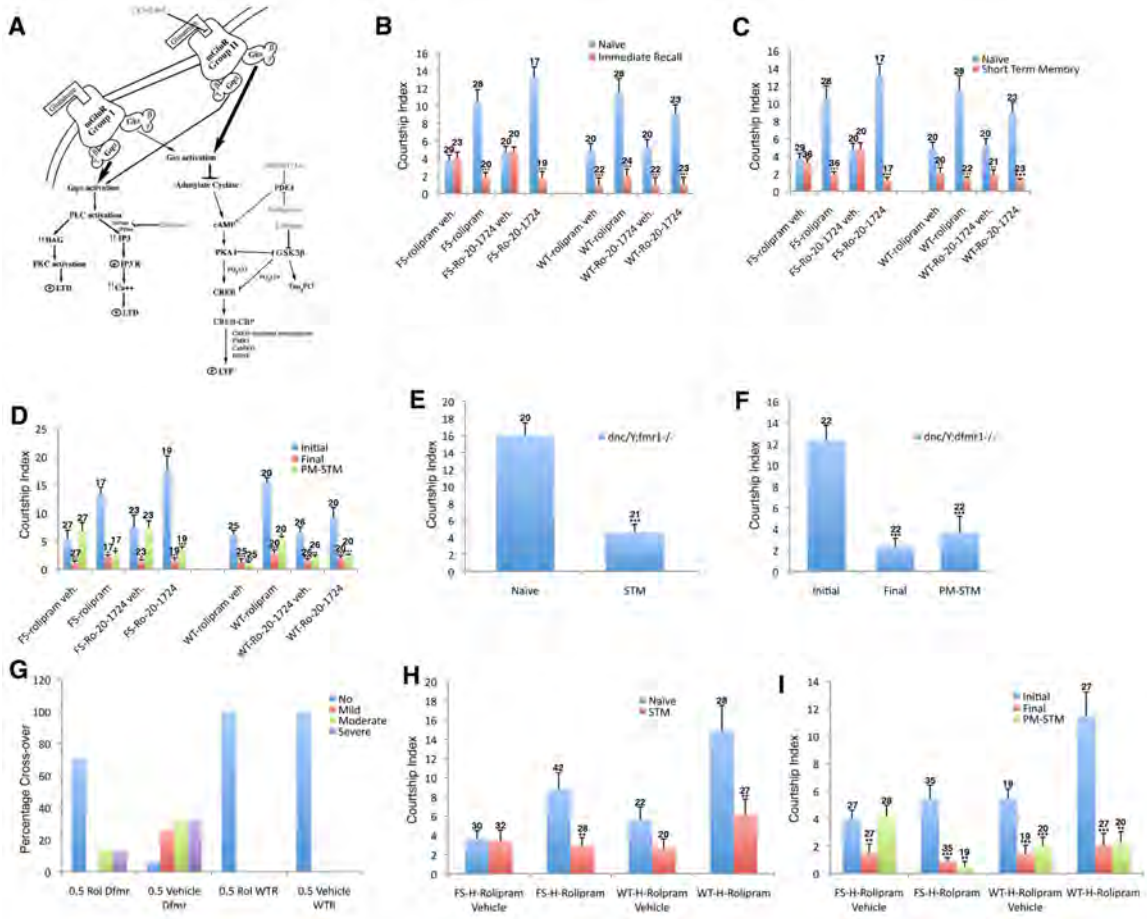

Figure 1. Rescue of memory in Fragile X flies treated with PDE-4 inhibitors.
A) The signal transduction pathway demonstrating the potential role for PDE-4 inhibitors in the treatment of Fragile X. The mGluR group I and mGluR group II signal transduction pathways are shown. Previously, it has been demonstrated that antagonizing or dampening the signaling of either of the mGluR pathways can rescue multiple phenotypes in the fly and mouse models of Fragile X including memory, audiogenic seizure and enhanced mGluR-LTD (McBride et al., 2005; Yan et al., 2005; Dolen et al., 2007; Choi et al., 2010; Choi et al., 2011). Additionally, lithium has demonstrated efficacy in rescuing cognitive abilities, audiogenic seizure and enhanced mGluR-LTD in fly and mouse models as well as in human patients (McBride et al., 2005; Berry-Kravis et al., 2008; Min et al., 2009; Choi et al., 2010; Yuskaitis et al., 2010b; Choi et al., 2011; Liu et al., 2011). As is displayed in the figure, PDE-4 also intersects in this signaling cascade. B) Immediate recall memory (0 minute post training) was measured in WT and FS flies that were administered vehicle control food, rolipram or Ro-20-1724 drug treatments. Training was performed by placing a naïve male with a previously mated female for a one-hour period. Memory represents a decrease in CI (courtship index) between the naive and testing period. Immediate recall memory was measured by placing a trained male with a virgin target immediately after training for a 10-minute courtship test interval. The mean CIs (±SEM) are plotted. The levels of significance are indicated as follows: *p <0.05; **p <0.01; ***p < 0.001. WT flies kept on vehicle, rolipram or Ro20-1724 demonstrate immediate recall memory. FS flies kept on vehicle fail to demonstrate memory. In contrast, FS flies treated with rolipram or Ro-20-1724 display immediate recall memory at 0 minutes after training. C) Short-term memory (60 minutes post training) was measured in WT and FS flies that were administered vehicle control food, rolipram or Ro-20-1724 drug treatments. Short-term memory was measured by placing a trained male in a holding chamber for 60 min (after a 1 hour training with a previously mated female), then subsequently placing him in a testing chamber with a mated female target for a 10 minute courtship interval. WT flies kept on vehicle, rolipram or Ro20-1724 demonstrate short-term memory. FS flies kept on vehicle fail to demonstrate short-term memory. In contrast, FS flies treated with rolipram or Ro-20-1724 display short-term memory at 60 minutes after training. D) Learning during training (LDT) and short-term (STM) (60 minute) memory were measured in WT and FS flies that were administered vehicle control food or rolipram or Ro-20-1724 drug treatments. WT flies kept on vehicle, rolipram or Ro20-1724 demonstrate a decrease in courtship during the final 10 minutes of training compared to the initial courtship, demonstrating learning during training. WT flies on all three treatments also demonstrate a significant decrease in courtship toward a pre-mated female target at 60 minutes after training (STM) compared to the initial courtship, demonstrating memory in this alternate memory testing paradigm. FS flies kept on vehicle display learning during training, but fail to demonstrate memory at 60 minutes after training. In contrast, FS flies treated with rolipram or Ro20-1724 display both learning during training and short-term memory at 60 minutes after training. E) Short-term memory (60 minute) was measured in Fragile X flies containing the dunce mutation, resulting in loss of function of the PDE-4 protein. Fragile X flies harboring the dunce mutation display short-term memory at 60 minutes after training. F) Short-term memory (60 minute) using the alternative paradigm was measured in Fragile X flies containing the dunce mutation. Fragile X flies harboring the dunce mutation display short-term memory at 60

minutes after training in the alternative paradigm. G) Mushroom body (MB) morphology was examined in WT and FS flies grown in food containing vehicle or a high dose of rolipram. The morphology of the MBs was performed as previously described in McBride et al., 2005; Michel et al., 2004 (Michel et al., 2004; McBride et al., 2005). The MBs in WT fly brains were normal after vehicle or high-rolipram treatment. Over 90% of the MBs in FS fly raised on vehicle control food displayed a range of cross-over defects, however significantly fewer MBs displayed cross-over defects when the FS flies were raised on food containing a high dose of rolipram. H) Short-term memory (60 minute) was measured in WT and FS flies that were administered vehicle control food or high dose rolipram treatments. WT flies kept on a high dose of rolipram demonstrate short-term memory, whereas those on vehicle did not display memory. FS flies kept on vehicle fail to demonstrate short-term memory. In contrast, FS flies treated with the high dose rolipram display short-term memory at 60 minutes after training. I) Learning during training (LDT) and short-term (STM)(60 minute) memory were measured in WT and FS flies that were administered vehicle control food or high dose rolipram treatments. WT flies kept on vehicle or high dose rolipram demonstrate a decrease in courtship during the final 10 minutes of training compared to the initial courtship, demonstrating learning during training. WT flies on both treatments also demonstrate a significant decrease in courtship at 60 minutes after training (STM) compared to the initial courtship, demonstrating memory. FS flies kept on vehicle display learning during training, but fail to demonstrate memory at 60 minutes after training. In contrast, FS flies treated with high dose rolipram display both learning during training and short-term memory at 60 minutes after training.

We next tested if the PDE-4 inhibitor rolipram could be effective in *Fmr1* KO mice. Since memory impairments have been difficult to replicate in this model, we chose to focus on a very reproducible electrophysiological phenotype. The most robust electrophysiological phenotype displayed by the Fragile X mouse model is exaggerated metabotropic glutamate receptor (mGluR)-dependent long-term depression (LTD) in the CA1 region of the hippocampus. We therefore decided to investigate the effects of PDE-4 inhibition on this form LTD in the mouse model. We initially tested the efficacy of chronic treatment in adulthood, since this was how we achieved rescue of memory in the fly model of Fragile X. In the current study mGluR-dependent LTD was induced by treating hippocampal slices with 100 μM DHPG for 10 minutes, which has been shown to stimulate mGluR-LTD in wild type mice (Huber et al., 2000; Huber et al., 2001; Choi et al., 2011).

Rolipram was chosen as the drug treatment to inhibit PDE-4 *in vivo* because of the high degree of selectivity and established dosing regiments in rats and mice (Barad et al., 1998; Gong et al., 2004). Rolipram or DMSO vehicle treatment was given to WT and *Fmr1* KO mice for 8 weeks beginning at 8-10 weeks of age. At the cessation of treatment, the mice were given a treatment-free hiatus for 3-5 weeks before being tested for DHPG-induced LTD. This was done to establish that transcriptional changes had occurred in the mice and to ensure that no drug was remaining in the system (Gong et al., 2004; Choi et al., 2011). In WT mice an 8 week treatment with DMSO vehicle had no effect on DHPG-induced mGluR-LTD, with depression of fEPSP slope values to 82.7 ± 2.8% and 83.4 ± 1.4% at 60 and 80 minutes, respectively, after induction (Figs. 2A, 3D and 3E). In contrast, WT mice that were chronically treated with rolipram demonstrated enhanced LTD of synaptic transmission at 60 and 80 minutes (67.4 ± 1.5% and 65.6 ± 2.4%, respectively) (Figs. 2A, 3D and 3E). There was no difference in basal synaptic transmission between WT mice treated with rolipram or DMSO vehicle (Fig. 2B). Also, there was no difference in paired-pulse facilitation (PPF) between WT mice treated with rolipram or DMSO vehicle suggesting that chronic rolipram treatment did not have an effect on presynaptic release mechanisms in the CA1 region of the hippocampus (Fig. 2C).

In *Fmr1* KO mice an 8 week treatment with DMSO vehicle had no effect on DHPG-induced mGluR-LTD, with LTD of 69.3 ± 1.4% and 71.1 ± 2.1% at 60 and 80 minutes after induction, which remained significantly enhanced compared to LTD in interleaved, age-matched, DMSO vehicle-treated WT mice at 60 and 80 minutes (82.7 ± 2.8% and 83.4 ± 1.4%; Fig. 3A, D and E). In contrast, *Fmr1* KO mice that were chronically treated with rolipram demonstrated abrogation of the enhanced mGluR-LTD endophenotype at 60 and 80 minutes after induction (87.6 ± 1.9% and 87.6 ± 1.9%; Fig. 3A D and E). Basal synaptic transmission and PPF were not significantly different between DMSO vehicle-treated and rolipram-treated *Fmr1* KO mice (Fig. 3B and C).

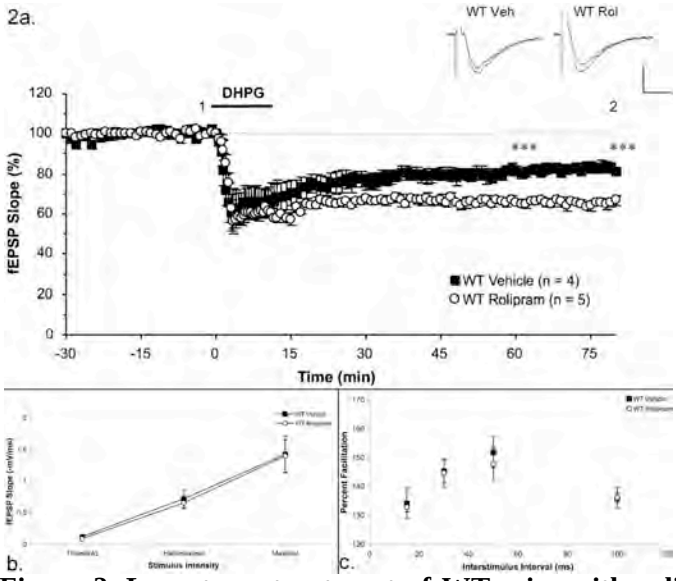

Figure 2. Long-term treatment of WT mice with rolipram enhances mGluR-LTD. A) Eight to ten week old WT mice were administered daily injections of rolipram for 8 weeks followed by a hiatus of 3-5 weeks. mGluR-LTD was induced by brief bath application of the mGluR agonist DHPG (100 μM, 10 min). Plotted are average fEPSP slopes (± SEM) as a percentage of average pre-induction baseline values. Representative traces of field potentials are from times indicated by the numbers on the graph (1 and 2). Calibration bars depict 1.5 mV and 5 ms. mGluR-LTD was significantly enhanced in rolipram-treated WT mice (n = 5 slices, 5 mice, open circles) compared to interleaved age-matched vehicle-treated WT mice (n = 4 slices, 4 mice, filled squares) at 60 minutes (WT vehicle: 82.7 ± 2.8%; WT rolipram: 67.4 ± 1.5%; p = 0.0001, ***) and at 80 minutes (WT vehicle: 83.4 ± 1.4%; WT rolipram: 65.6 ± 2.4%; p = 0.0001, ***) post-induction. B) Basal synaptic transmission is not affected by chronic rolipram treatment in WT mice. Mean evoked fEPSP slopes (± SD) are plotted at three different stimulus intensities. Synaptic responses at threshold, half-maximal and maximal stimulus intensities were not significantly different between rolipram-treated WT mice (n = 5 slices, 5 mice, open circles) and interleaved age-matched vehicle-treated WT mice (n = 4 slices, 4 mice, filled squares). C) Paired-pulse facilitation (PPF) in WT mice after chronic rolipram treatment (n = 5 slices, 5 mice, open circles) and interleaved age-matched vehicle-treated WT mice (n = 4 slices, 4mice, filled squares) was not different. Synaptic responses to paired stimulation were evoked at interstimulus intervals ranging from 15 ms to 100 ms. Plotted are the mean percent facilitation (± SD), as determined by calculating the ratio of the second fEPSP slope to the first fEPSP slope.

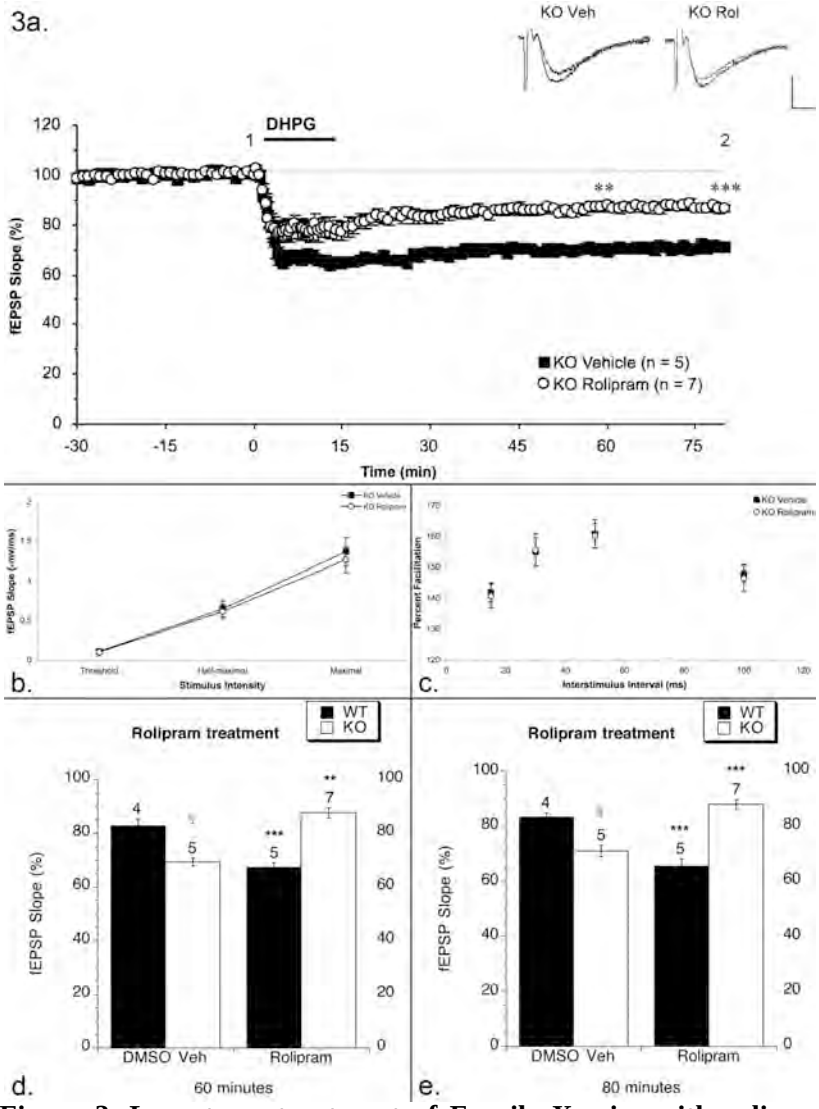

Figure 3. Long-term treatment of Fragile X mice with rolipram. A) Eight to ten week old Fmr1 KO mice were administered daily injections of rolipram for 8 weeks followed by a hiatus of 3-5 weeks. LTD was induced, measured and plotted as described in Figure 2 legend. mGluR-LTD was significantly enhanced in vehicle-treated Fmr1 KO mice (n = 5 slices, 5 mice, filled squares) compared to interleaved age-matched vehicle-treated WT mice (Figure 2; n = 4 slices, 4 mice, filled squares) at 60 minutes (WT vehicle: 82.7 ± 2.8%; Fmr1 KO vehicle: 69.3 ± 1.4%; Panels 3A and 3D, p = 0.0001) and at 80 minutes (WT vehicle: 83.4 ± 1.4%; Fmr1 KO vehicle: 71.1 ± 2.1%; Panels 3A and 3E, p = 0.0003) post-induction. Chronic treatment of Fmr1 KO mice with rolipram (n = 7 slices, 7 mice, open circles) abrogated the enhanced mGluR-LTD phenotype compared to vehicle-treated Fmr1 KO mice at 60 minutes (Fmr1 KO vehicle: 69.3 ± 1.4%; Fmr1 KO rolipram: 87.6 ± 1.9%; Panel 3D, p = 0.001, **) and at 80 minutes (Fmr1 KO vehicle: 71.1 ± 2.1%; Fmr1 KO rolipram: 87.9 ± 1.9%; p = 0.0001, ***) post-induction. B) Mean evoked fEPSP slopes (± SD) are plotted at three different stimulus intensities. Synaptic responses at threshold, half-maximal and maximal stimulus intensities between rolipram-treated Fmr1 KO mice (n = 7 slices, 7 mice, open circles) and interleaved age-matched vehicle-treated Fmr1 KO mice (n = 5 slices, 5 mice, filled squares) were not different. C) PPF, evoked as describe in Figure legend 2, between rolipram treated Fmr1 KO mice (n = 7 slices, 7 mice, open circles) and interleaved age-matched vehicle-treated Fmr1 KO mice (n = 5 slices, 5 mice, filled squares) was not different. Synaptic responses to paired stimulation were evoked at interstimulus intervals ranging from 15 ms to 100 ms. Plotted are mean percent facilitation (± SD), as determined by calculating the ratio of the second fEPSP slope to the first fEPSP slope. D-E) DHPG-LTD in WT and Fmr1 KO mice treated with vehicle or rolipram at 60 or 80 minutes after induction. Two-way ANOVA was performed: ** represents p < 0.001; *** represents p < 0.0001. The § indicates a significant difference between WT and Fmr1 KO mice on vehicle treatment (p = 0.0001) at 60 minutes and (p = 0.0003) at 80 minutes. The asterisks represent significance with respect to vehicle treatment within the same genotype. The number above each bar denotes the n.

mGluR-LTD was examined in hippocampal slices from untreated WT and *Fmr1* KO mice (at 20-23 weeks of age) after acute bath application of rolipram at a concentration that is within the range observed in the brain of mice during chronic treatment (Barad et al., 1998; Gong et al., 2004). Acute experiments differ from chronic treatment in that a drug effect is examined on the unadulterated state of the synapse. Signaling at the synapse in

WT mice is presumed to be set up to maintain a homeostatic balance leading to optimal LTD in response to appropriate synaptic stimulation, an inverted U-model of signaling with regard cAMP (Sato et al., 2004). In the inverted U-model of homeostatic balance with regard to cAMP signaling, the optimal level of cAMP will allow for proper signaling and memory formation, whereas hypoactive cAMP signaling or hyperactive cAMP signaling will lead to memory impairment. The classical example of this with regard to memory was first provided in Drosophila where the rutabaga mutation leads to hypoactive cAMP signaling and the dunce mutation leads to hyperactive cAMP signaling and both result in memory impairment. Consistent with this supposition we found that acute treatment with rolipram had no effect on LTD in WT mice at 80.1 ± 0.7% and 80.1 ± 1.5% at 60 and 80 minutes after induction (Fig. 4A, C and D). Similar acute treatment with DMSO vehicle also had no effect on DHPG-induced mGluR-LTD in WT mice, which is 77.9 ± 2.4% and 80.2 ± 2.9% of average pre-induction baseline values at 60 and 80 minutes post-induction (Fig. 4A, C and D). These finding suggest that under this set of conditions the signaling system may prevent overactive cAMP signaling from altering the magnitude DHPG-induced mGluR-LTD in WT mice.

mGluR-LTD remained enhanced in *Fmr1* KO mice upon bath application of DMSO vehicle at 72.2 ± 1.0% and 72.0 ± 1.2% of baseline values at 60 and 80 minutes after induction (Fig. 4B, C and D). In contrast, acute bath application with rolipram eliminated the enhancement
of mGluR-LTD, with fEPSP slope values of 81.3 ± 1.9% and 81.9 ± 1.8% relative to baseline at 60 and 80 minutes after induction (Fig. 4B, C and D). This demonstrated that acute increases in cAMP can restore mGluR-LTD to WT levels, indicating that there is a role for cAMP in the acute regulation of mGluR-LTD in *Fmr1* KO mice.

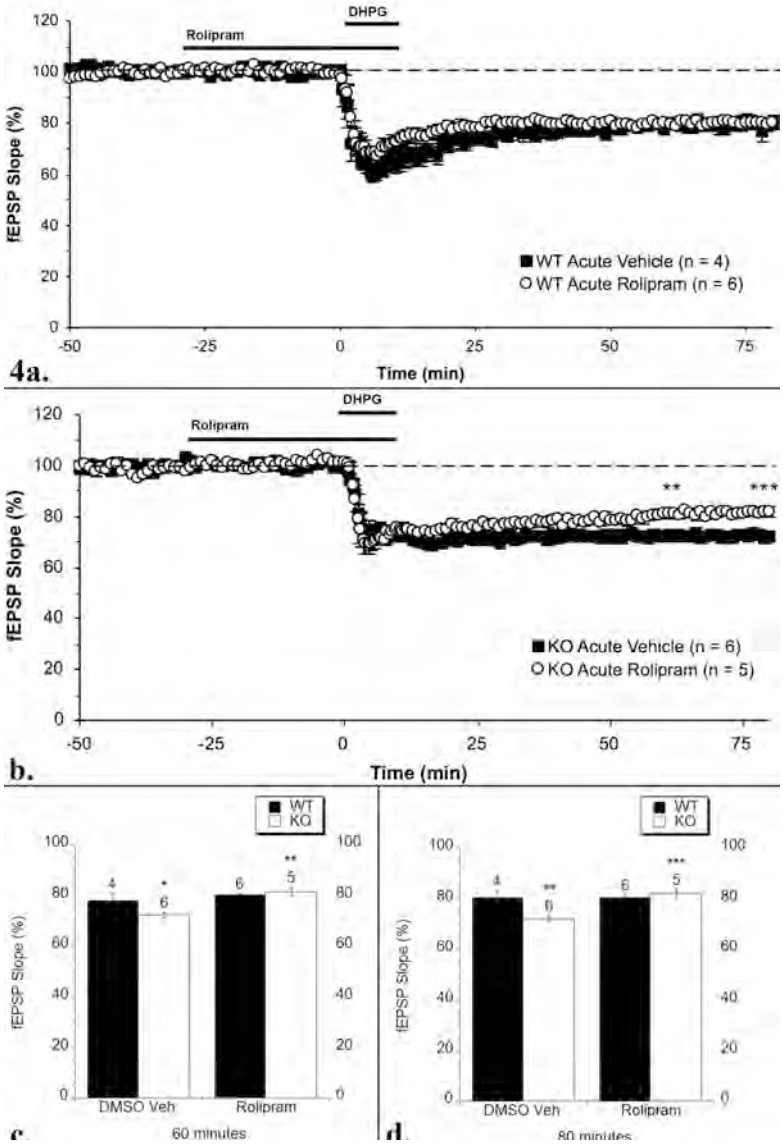

Figure 4. Differential effects of acute rolipram treatment in WT vs Fmr1 KO mice.
A-B) Acute bath application of rolipram in WT mice. Plotted are average fEPSP slope values (± SEM) as a percentage of average pre-induction baseline values. A) WT mice were acutely treated with rolipram (n = 6 slices, 6 mice, open circles) or with DMSO vehicle alone (n = 4 slices, 4 mice, filled squares) at 60 minutes (WT acute vehicle: 77.9 ± 2.4%; WT acute rolipram: 80.1 ± 0.7%) and at 80 minutes (WT acute vehicle: 80.2 ± 2.9%; WT acute rolipram: 80.1 ± 1.5%) post-induction. B) Acute application of rolipram to slices from Fmr1 KO mice (n = 5 slices, 5 mice, open circles) compared to acute vehicle-treated Fmr1 KO mice (n = 6 slices, 6 mice, filled squares) at 60 minutes (Fmr1 KO acute vehicle: 72.2 ± 1.0%; Fmr1 KO acute rolipram: 81.3 ± 1.9%; Panel 4C, p = 0.0002) and at 80 minutes post-induction (Fmr1 KO acute vehicle: 72.0 ± 1.2%; Fmr1 KO acute rolipram: 81.9 ± 1.8%; Panel 4D, p = 0.0001). C-D) The graphs illustrate DHPG-LTD in WT and Fmr1 KO mice treated with vehicle or rolipram at 60 or 80 minutes after induction. Two-way ANOVA was performed: ** represents $p < 0.001$; *** represents $p < 0.0001$. The § indicates a significant difference between WT and Fmr1 KO mice on vehicle treatment (p = 0.02) at 60 minutes and (p = 0.0015) at 80 minutes. The asterisks represent significance with respect to vehicle treatment within the same genotype. The number above each bar denotes the n. Acute rolipram treatment significantly reduces mGluR-LTD in Fmr1 KO mice, in contrast no effect of treatment is seen in WT mice.

Reportable outcomes: The above-described studies have been submitted for publication and are currently under revision based on reviewers comments.

Key Research Accomplishments:

We have completed Task 1.
Task 1. Completion of testing cAMP-PDE antagonists on the *Drosophila* fragile X model.
1a. Test naïve courtship, learning during training (LDT), and memory (STM) in *dfmr1* mutant and control flies treated with drug or vehicle with continuous, development alone or adulthood alone**.**
1b. Genetically validate the results obtained with the PDE-4 inhibitors.

Task 3. We have completed testing with an additional HDAC inhibitor (TSA) and have validated the results we have obtained with sodium butyrate. These results provide us with additional evidence to pursue the HDAC inhibitor treatments that are outlined in Task 14. These tasks will be initiated after the tasks 12 and 13 are completed.

Task 13. Examination of the effect of treating *FMR1* KO and control mice with PDE-4 inhibitors.
13a. Obtain *FMR1* KO and control mice that have been aged, treated with drug (rolipram, Ro 20-1724) or vehicle and then put on treatment hiatus, to perform electrophysiological analysis
13d. Perform electrophysiological and EEG analysis on *FMR1* KO and control mice that are treated with drug or vehicle

Ongoing tasks:

Task 2-The outcrossing of *Gsk-3beta*, *IPPase*, *InsP3R*, *Rheb*, *S6K* mutant stocks and the transgenic stocks *UAS-AMPK*, *UAS-4EBP* is ongoing to prepare these stocks for behavioral testing.

Task 4. Test PI3K antagonists on the *Drosophila* fragile X model.
4a. Test naïve courtship, learning during training (LDT), and memory (STM) in *dfmr1* mutant and control flies treated with drug or vehicle with continuous, development alone or adulthood alone.
4b. Genetically validate the results obtained with the PI3K inhibitors.
4c. Perform biochemical analysis to determine effects of PDE-4 inhibition on PI3K and Akt activity and smRP6 levels.

Task 5. Test Gsk-3Beta antagonists on the *Drosophila* fragile X model.
5a. Test naïve courtship, learning during training (LDT), and memory (STM) in *dfmr1* mutant and control flies treated with drug or vehicle with continuous, development alone or adulthood alone.
5b. Genetically validate the results obtained with the Gsk-3Beta inhibitors.
5c. Perform biochemical analysis to determine effects of Gsk-3Beta inhibition on PI3K and Akt activity and smRP6 levels.

Conclusions:

The overall objective of the work we have accomplished so far was to examine the efficacy of pharmacologically inhibiting PDE-4 activity to correct synaptic plasticity impairments in the fly and mouse models of Fragile X syndrome. The *Drosophila* Fragile X model recapitulates the most debilitating aspect of the disease in humans, namely impaired cognitive function. In our further dissection of the proteins involved in the mGluR signaling cascade, we identified PDE-4 as a potential substrate whose inhibition may be beneficial in restoring proper intracellular signaling in the Fragile X model (Fig. 1A). Based on the fly data, tissue culture work, the mouse model and samples from humans afflicted with Fragile X syndrome, we speculated that cAMP levels are suppressed (Berry-Kravis and Sklena, 1993; Berry-Kravis et al., 1995; Berry-Kravis and Ciurlionis, 1998; McBride et al., 2005; Kelley et al., 2007). PDE-4 inhibition should increase cAMP signaling by preventing the breakdown of cAMP that is produced during synaptic stimulation. Fragile X flies chronically treated in adulthood with PDE-4 inhibitors, or with genetically reduced levels of PDE-4, demonstrated intact immediate recall and short-term memory, validating PDE-4 inhibition as a potential novel therapeutic target for the treatment of synaptic plasticity impairments in Fragile X. This finding adds to the growing body of literature demonstrating that pharmacologic treatment initiated in adulthood may have efficacy for the treatment of cognitive disorders that are already present in childhood as was first demonstrated in animal models of Fragile X and Neurofibromatosis type 1 in 2005 (Li et al., 2005; McBride et al., 2005; for review see Raymond and Tarpey, 2006; or Walsh et al., 2008).

In summary or work demonstrates that PDE-4 inhibition is a novel therapeutic target for the treatment of Fragile X. Prior to this work, it has only recently been demonstrated that enhanced LTD in the Fragile X model could be abrogated by chronic pharmacologic treatment (Choi et al., 2011). Equally as important is the demonstration that treatment in adulthood alone can rescue the phenotype, meaning that the phenotype is not irreversibly determined by pathogenic developmental circuitry. These findings urge the need for further exploration of PDE-4 inhibition as a potential therapy in Fragile X patients and in animal models of fragile X. Additionally, this work is a stepping stone for the field to begin a further pharmacologic dissection of the pathogenic signaling leading to aberrant LTD in the Fragile X model mouse, with the hope of these findings allowing the treatment of patients afflicted with Fragile X.

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